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Characterization of humic acid size fractions by SEC and MALS

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Abstract

Latahco silt-loam humic acid was separated on a preparatory scale by size exclusion chromatography (SEC) on a gravity-fed Sepharose column. Four fractions from this separation were collected and further analyzed, along with whole humic acid, by high-performance SEC coupled with a multi-angle light scattering (MALS) detector. A detailed characterization of the radius of gyration across the elution profile of each fraction by MALS demonstrated that the preparative column generally separated the humic acid according to differences in molecular size. Furthermore, a comparison of elution profiles obtained on the whole humic acid with UV and refractive index detectors revealed the presence of a non-aromatic low molecular weight component. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Humic acid; size-exclusion chromatography; HPSEC; multi-angle light scattering

1. Introduction

Previous work has indicated that molecular size is an important determinant in the associative behavior of humic acid (HA) dissolved in aqueous solution (Hassett and Anderson, 1982; Engebretson and von Wandruszka, 1997). Considerations of size are especially relevant to the detergent model of HA, which holds that these natural polymers have a pronounced surfactant character and are apt to aggregate spontaneously (Wershaw et al., 1986; Engebretson and von Wandruszka, 1994). The structures thus formed, often referred to as pseudomicelles, are thought to be akin to the micelles of other amphophilic species such as synthetic detergents, albeit subject to constraints related to their greater molecular weight and polydispersity. In this scenario, humic polymers undergo both intra- and intermolecular aggregation (Ragle et al., 1997; von Wandruszka et al., 1997). The former involves the coiling and folding of longer HA chains, resulting in pseudomicellar domains with relatively hydrophobic cores and hydrophilic surfaces. It is intuitively obvious that molecular size plays a role in associative processes of this type, and evidence for this was obtained in a previous study (Engebretson and von Wandruszka, 1997).

Size exclusion chromatography (SEC) is the most convenient and widely used technique for the determi-

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nation of molecular weight distributions in polymeric materials, both synthetic and natural. SEC separates dissolved macromolecules according to their size, or more specifically, their hydrodynamic volume. Molecular weights are typically estimated with the use of polymer standards, assuming that the relationship between size and molecular weight is the same for the standards and samples being analyzed. The size-based separation of charged species such as HAs by SEC is subject to uncertainty because of exclusion effects, which consist of both attractive and repulsive electrostatic interactions that perturb the separation mechanism. It is the aim of this study to evaluate the applicability of SEC for preparative HA separations, by assessing the integrity of the size fractions obtained for a typical HA on a Sepharose preparatory-scale size exclusion chromatography (PSEC) column. To this end, the fractions isolated from these chromatographic runs were further separated and analyzed by high-performance SEC (HPSEC) with detection by multi-angle light scattering (MALS). The MALS detector provides an absolute measure of molecular size as components elute from the SEC column, giving estimates superior to those obtained from comparison with standards.

Although MALS can also be used to measure the molecular weight of macromolecules, such determinations were precluded in this study by the fluorescence of the sample. Molecular weights are calculated in MALS by extrapolating the angular dependence of light scattering to zero angle and the values are therefore strongly biased by fluorescence. However, since this emission is isotropic, calculations of molecular size are not affected, since they are based on the slope of the plot of intensity versus angle for scattered radiation.

2. Materials and methods

2.1. Humic acid and preparatory-scale LC

The collection, storage, processing and chemical characterization of Latahco silt loam (Argiaquic Xeric Argialbolls), as well as the metal and ash content of the humic acid (LSLHA) obtained from this soil have previously been reported (Engebretson and von Wandruszka, 1994). Extraction and deashing was carried out according to the procedure published by the International Humic Substance Society (IHSS, 1985), except that the material was stored for approximately 1 month in the dark at 4°C prior to the deashing step. Solutions of 100 ppm humic acid were prepared with minimum sonication, at an original pH adjusted to 10 with 1 M NaOH. No subsequent pH adjustments were made and the solutions were left at room temperature until a constant pH was reached, which took 1–2 weeks. The equilibrium pH was in the range 7.4–7.8. Water used in all solutions was deionized and treated with a 0.22 mm Millipore filter system to a resistivity of 18 MΩ cm.

PSEC separations of LSLHA were carried out with a 45-cm gravity fed column, operated at constant head pressure, packed with Sepharose 6B-100 stationary phase (Sigma) and eluted with a pH 9 Tris buffer [tris(hydroxymethyl)amino methane]. This stationary phase had a nominal molecular weight range of 10,000–4,000,000 g mol^{-1} based on globular protein standards and 10,000–1,000,000 g mol^{-1} based on dextran standards. Tris was obtained from Sigma and prepared as recommended by Cameron et al. (1972). The eluent flow rate was 1.5 ml/min and elution was monitored with a 254-nm LC detector (Gilson).

2.2. HPSEC of LC fractions

The HPSEC separation was carried out with a Supelco (Bellefonte, PA) TSKTM G5000 PW column of 30 cm length and 7.5 mm internal diameter. The nominal molecular weight range of this column, based on dextran standards, was 50,000–7,000,000 g mol^{-1}. The flow rate used in the separation was 0.5 ml/min. The MALS detector was a Dawn Model DSP from Wyatt Technology (Santa Barbara, CA). The refractive index detector was an Erma Model ERC-75 1 5A from FFFractionation, LLC (Salt Lake City, UT). A Linear Model 200 variable wavelength detector from Alltech Associates (Deerfield, IL) was used at a wavelength of 280 nm. The injection volume of all samples was 100 μl. The concentration of the unfractionated (whole) humic acid sample used was 375 μg/ml. Two different mobile phases were used in the HPSEC separations: (1) to compare whole humic acid with the excluded peak obtained from preparatory LC, the mobile phase had a pH of 7.9 and contained 0.05 M Tris, 0.0268 M HNO_3 and 0.00308 M NaN_3; (2) the separation of other LC fractions was carried out with a mobile phase consisting of 0.1 M phosphate buffer (pH 6.8) prepared with Na_2HPO_4 and KH_2PO_4. The whole humic acid was also analyzed in the phosphate buffer.

2.3. Determination of molecular size by MALS

The theory of light scattering can be found in several textbooks on the subject (e.g. Huglin, 1981). In MALS, the intensity of scattered laser light emitted by sample molecules is measured at several different angles (θ) simultaneously. The weight-average molecular weight (M_w) and z-average root-mean-square radius (r_g^2) are calculated using Eq. (1):

$$\frac{Kc}{R_\theta} = \frac{1}{M_w P(\theta)} + 2A_2 c + \ldots \quad (1)$$

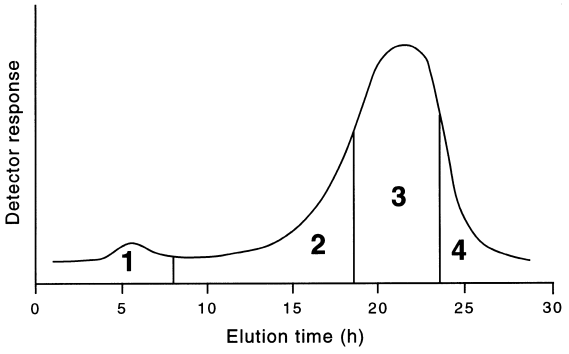

Fig. 1. Elution profile of whole LSLHA separated on a preparatory-scale Sepharose column with Tris eluent.

where K is the light scattering constant, c is the concentration, A_2 is the second virial coefficient, R_θ is the excess Rayleigh ratio and $P(\theta)$ is the form factor, which describes the angular variation of the scattering by

$$P(\theta) = 1 - \alpha_1 \sin^2(\theta/2) + \alpha_2 \sin^4(\theta/2) - \ldots \quad (2)$$

The coefficients α_i depend on the structure of the molecule and are determined by fitting the collected light-scattering data to various angles. Coefficient α_1 is related to r_g^2 by

$$\alpha_1 = \left(\frac{4\pi n_0}{\lambda_0}\right)^2 \frac{r_g^2}{3} \quad (3)$$

where n_0 is the refractive index of the solvent and λ_0 is the wavelength of the incident light beam.

By combining MALS with a high-resolution separation technique such as SEC, the eluting concentrations are generally small enough that the second term in Eq. (1) can be ignored, which simplifies the calculations. Thus, for a particular eluting slice, R_θ is proportional to $P(\theta)$ and the quantity KcM_w is merely a proportionality factor. Measurement of R_θ at various angles can then be fit to Eq. (2) using standard nonlinear fitting techniques. This yields values of r_g^2 at each elution slice independent of sample concentration, molar mass, or separation mechanism. Because it is isotropic, fluorescent radiation does not interfere with the measurement r_g^2 by MALS.

The data manipulations described here were carried out by Astra Version 4.5 software provided by Wyatt Technology.

3. Results and discussion

The LSLHA used in this study was, like all HAs, a highly polydisperse material. In the PSEC separation, this circumstance manifested itself through broad chromatograms showing a virtually continuous distribution. Fig. 1 shows the elution profile of whole LSLHA separated on the Sepharose PSEC column. The vertical lines indicate the boundaries of fractions collected for further analysis by HPSEC-MALS. Fraction 1 represents the totally excluded peak, containing species too large to be retained by the PSEC column. The HPSEC separation, recorded by the 90° MALS detector, of this fraction and the whole LSLHA, are illustrated in Fig. 2. Fig. 2 also includes a plot of the radius of gyration across each elution pro-

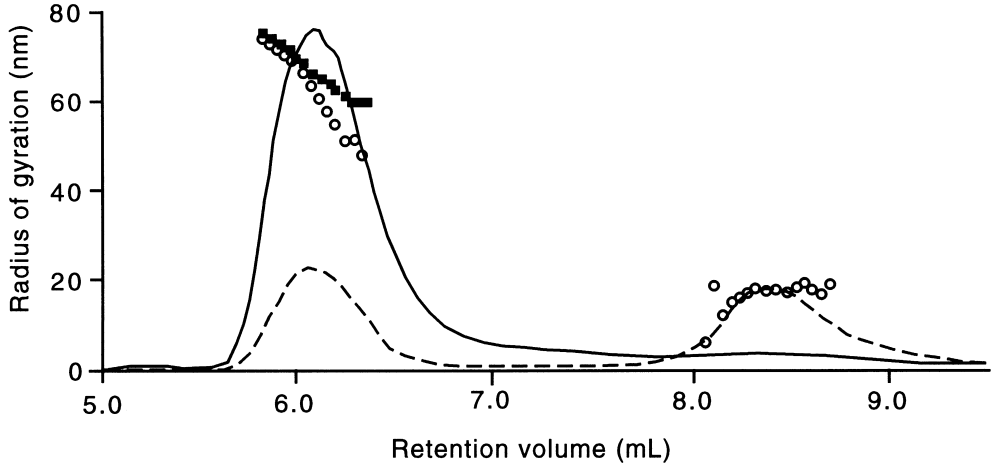

Fig. 2. Radius of gyration and corresponding HPSEC-MALS elution profiles of whole LSLHA and the fraction totally excluded by the Sepharose column. - - - ○ - - -, whole LSLHA; – ■ –, totally excluded fraction.

file. As expected, the radius generally decreased with retention volume (V_r). The profile of the whole LSLHA sample had two peaks, one centered at 6 ml and the other at 8.5 ml. The excluded fraction, by contrast, consisted of one tailing peak with a maximum at 6 ml; very little material eluted after 7 ml. The radii calculated by MALS indicated that material eluted at 6 ml was similar for both samples. These results confirm the isolation of high molecular weight material in fraction 1 from the Sepharose column. Regarding the whole LSLHA sample, the increase in size for material eluting from the HPSEC column between 8 and 9 ml was unexpected and may indicate other interactions with the column in addition to size exclusion. Nevertheless, the molecular size of this material was smaller than that eluted at 6 ml, confirming that the preparatory separation proceeded according to size.

Previous studies (Schimpf and Petteys, 1997; Schimpf and Wahlund, 1997) have demonstrated significant changes in the hydrodynamic behavior of humic materials with subtle changes in solvent parameters, including pH, ionic strength and electrolyte composition. These changes can affect the elution times in SEC, making it difficult to obtain consistent information on size or molecular weight without an absolute measurement technique such as MALS. To illustrate this phenomenon, Fig. 3 compares the elution profile of LSLHA in the Tris buffer with that obtained using a 0.1 M phosphate buffer (pH 6.8) as the mobile phase. Values of r_g^2 obtained by MALS on the eluting fractions are also contained in Fig. 3. Note that the peak centered at 8.5 ml in Tris shifted to 9 ml in phosphate, but r_g^2 is unchanged. Evidently, the smallest pores in the packing material are only accessible in phosphate buffer. Exclusion of this fraction of the humic material from the smallest pores in TRIS reduces its retention volume.

Regarding the early eluting material, note that an additional component appears in phosphate buffer. The size of this new component is lies between the two components measured in Tris. This new component is either adsorbed to the column in Tris or its appearance is a consequence of differences in aggregation behavior in the two buffers. For example, it is possible that the two partially resolved components in phosphate buffer consist of aggregated and non-aggregated high molecular weight material, whereas the early eluting material in Tris is completely aggregated.

Fig. 4 compares the HPSEC elution profiles of the whole LSLHA as recorded by MALS (90°), refractive index (RI) and UV (280 nm) detectors. In contrast to the MALS signal, the RI and UV detectors responded poorly to material eluting between 5.5 and 7 ml. On the other hand, these two detectors responded relatively strongly to components eluting between 7 and 8 ml. This reversal in detector sensitivity is not unusual, as light scattering increases sharply with molecular weight. The RI detector, also responded to several late eluting components that gave very weak responses from the other two detectors. This material probably consisted of small molecules without aromatic chromophores. This comparison of detector responses illustrates the importance of using multiple detectors in the characterization of complex materials that vary in both molecular weight and chemical composition.

Three additional fractions were collected across the primary peak in the elution profile of the Sepharose column (fractions 2–4, Fig. 1). Each fractions was further characterized by HPSEC using phosphate buffer as the mobile phase. The results are summarized in

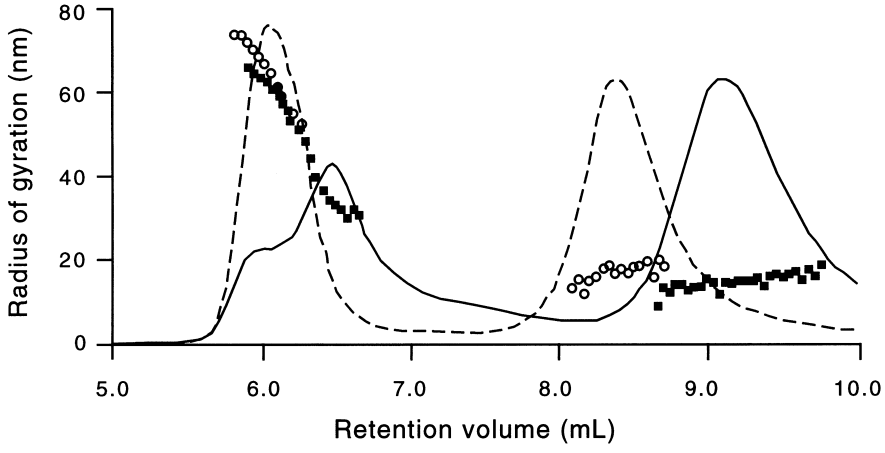

Fig. 3. Molecular weights and corresponding HPSEC-MALS elution profiles of whole LSLHA eluted with Tris and phosphate buffers. - - - ○ - - -, Tris; – ■ –, phosphate.

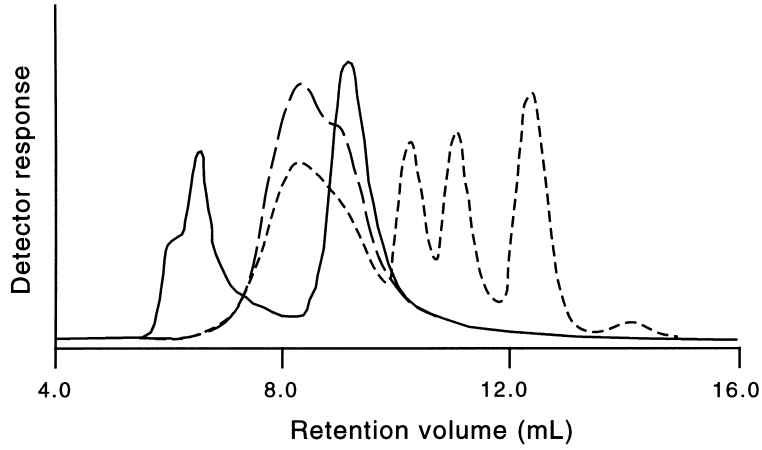

Fig. 4. Comparison of HPSEC elution profiles of whole LSLHA as recorded by a MALS 90° detector (———), a UV detector (— — —), and a RI detector (- - -).

Fig. 5, which shows that the elution profiles recorded by the RI and UV detectors were significantly different. This can again be ascribed to the fact that a 280-nm UV detector is especially sensitive to HA fractions containing aromatic chromophores and less so to aliphatic fractions. The response of the RI detector is more universal, but does vary with the chemical composition of the eluting material. Since humic acids differ widely in that regard, the relative magnitude of the RI signal cannot be used to quantify the mass distribution of eluting material. Nevertheless, the RI profile should be considered a better representation of the sample in its entirety. Furthermore, comparison of the two profiles allows for a qualitative assessment of the aromatic and aliphatic content of the eluting sample. The RI profile shows that there was more early eluting

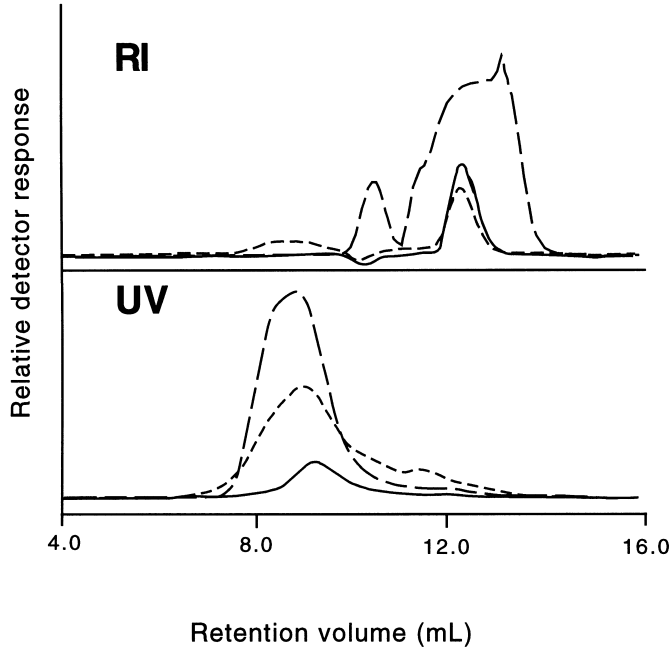

Fig. 5. Comparison of HPSEC elution profiles of fractions 2–4 from the Sepharose column as recorded by the RI and UV detectors. — — —, fraction 2; - - -, fraction 3; ———, fraction 4.

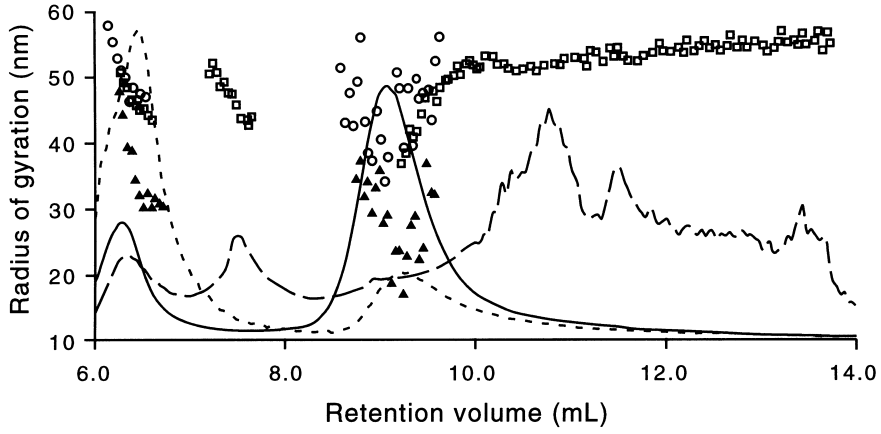

Fig. 6. Radius of gyration and corresponding HPSEC-MALS elution profiles of fractions 2–4 from the Sepharose column. — — — □ — — —, fraction 2; - - - ○ - - - fraction 3; – ▲ –, fraction 4.

material in fraction 3 than in fraction 4, while fraction 2 contained more late eluting species. The RI profile of fraction 2 also shows a peak between 10 and 11 ml, which did not appear in fractions 3 and 4. Compared to the RI detector, the UV detector recorded more early eluting material. Fractions 2 and 4 contained components eluting between 8 and 10 ml, which were not detected by RI. The presence of humic acid peaks recorded only by certain detectors has been reported previously (Wagoner and Christman, 1998).

The situation becomes clearer when the MALS data shown in Fig. 6 are considered. The size of the late eluting material in fraction 2 ($V_r > 9$ ml) was found to be relatively large and increased with volume. This indicates that its retention in the HPSEC column was based on adsorption, rather than exclusion, mechanisms. Similar profiles were obtained in Tris buffer (data not shown). When this material is considered together with the early eluting material in fraction 2 ($V_r = 6$–7 ml), it becomes apparent that fraction 2 did in fact contain a greater amount of large molecules than either fraction 3 or 4. Furthermore, comparing the UV and RI signals of fraction 2 (Fig. 5) suggests that aromatic components were separated in the HPSEC column by an exclusion mechanism while aliphatic components tended to adsorb to the column material.

The cumulative size distributions of fractions 2–4 are shown in Fig. 7. These values were calculated directly from the light scattering data by Astra software, without implementation of any smoothing pro-

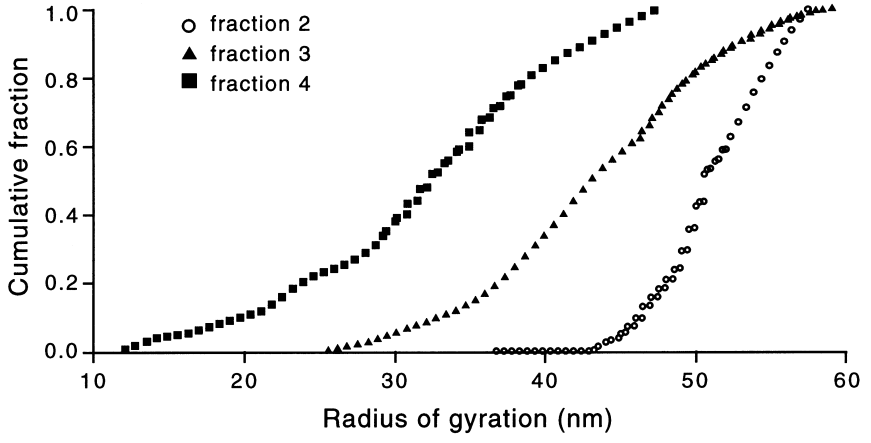

Fig. 7. Distributions of the radii of gyration of fractions 2–4 from the Sepharose column.

cedures. Despite the noise in calculated radii, particularly for peaks eluting between retention volumes of 8.5 and 9.5 ml (see Fig. 6), it is clear from the data in Fig. 7 that molecular size decreased from fraction 2 through fraction 4.

4. Conclusion

The evaluation of HA size separation presented here is based on LSLHA only. In view of the general similarity of HA elution profiles, however, it is likely that the observations concerning molecular sizes obtained with the columns used in this study can be extended to other HAs.

Acknowledgements

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